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ORIGINAL ARTICLE

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The angiogenesis inhibitor NM-3 is active against human NSCLC xenografts alone and in combination with docetaxel

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Abstract The novel isocoumarin 2-(8-hydroxy-6-methoxy-1-oxo-1 *H*-2-benzopyran-3-yl) propionic acid (NM-3) has completed phase I clinical evaluation as an orally bioavailable angiogenesis inhibitor. NM-3 directly kills both endothelial and tumor cells in vitro at low mM concentrations and is effective in the treatment of diverse human tumor xenografts in mice. The present work has assessed the activity of NM-3 against human non-small-cell lung cancer (NSCLC) cells when used alone and in combination with docetaxel. The results demonstrate that NM-3 decreases clonogenic survival of NSCLC cells at clinically achievable concentrations. The results also demonstrate that NM-3 is effective in the treatment of NSCLC (A549, NCI-H460) tumor xenografts in mice. Moreover, NM-3 potentiated the antitumor activity of docetaxel against NSCLC xenografts without increasing toxicity. Our findings indicate that NM-3 may be effective alone or in combination with docetaxel in the treatment of patients with NSCLC.

Keywords NM-3 · Docetaxel · NSCLC · Tumor xenografts · Anti-angiogensis

Introduction

NM-3 is an isocoumarin derivative that has completed phase I evaluation as a novel anti-angiogenesis agent [1, 2]. NM-3 was selected for clinical evaluation based on its oral bioavailability, favorable toxicity profile in preclinical studies and dose-dependent anti-angiogenic activity in the mouse dorsal air sac model [3]. NM-3 is active as a single agent against Lewis lung carcinomas and human tumor xenografts in mice [4]. In addition, NM-3 potentiates the antitumor activity of radiotherapy without an increase in toxicity [4]. Other studies have demonstrated that NM-3 increases the antitumor effects of taxol and cyclophosphamide against human breast and prostate tumor xenograft models [5]. These effects of NM-3 are attributable, at least in part, to the induction of endothelial cell lethality. In this regard, human umbilical vein endothelial cells (HUVECs) are killed by in vitro exposure to low mM concentrations of NM-3 [4]. NM-3 also has direct effects against human carcinoma cells [6]. At clinically achievable concentrations (100 μg/ml), NM-3 inhibits clonogenic survival of human carcinoma and multiple myeloma cells by increasing levels of reactive oxygen species (ROS) and sensitizing cells to the induction of apoptosis [6]. These findings have indicated that NM-3 may be effective in targeting both the tumor and its vasculature.

The present work has examined the effects of NM-3 on human NSCLC cells. The results show that NM-3 inhibits the survival of NSCLC cells in vitro and growth of NSCLC xenografts in mice. We also show that NM-3 potentiates the antitumor activity of docetaxel in in vivo NSCLC tumor models.

Materials and methods

Cell culture

Human A549, Calu-6, NCI-H23 and NCI-H460 NSCLC cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere at 37°C. The cells were treated with NM-3 dissolved in Mg2+- and Ca2+-free phosphate-buffered saline (PBS) and 10 mM HEPES, pH 7.4. Docetaxel (Aventis Pharmaceuticals) was dissolved in 13% ethanol in H_2O and diluted in PBS.

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Cell viability and clonogenic survival assays

Cells were plated in 96-well plates for 24 h and then NM-3 and/or docetaxel were added to the culture medium. After incubation for 72 h, the number of cells was determined by the MTT assay. For combination treatment, NM-3 was added to the culture 2 h before docetaxel. In colonogenic assays, cells were plated in 60-mm dishes for 8 h before adding NM-3. Cells were incubated for 11–15 days and then stained with crystal violet. Colonies those consisted of 50 or more cells were counted.

Tumor xenograft models

A549 cells were suspended in serum-free RPMI1640 medium and injected into the right flank (6×10^6 cells/mouse) of female nude NCRNU mice. NCI-H460 tumor fragments (30–40 mg) were injected subcutaneously into the right axillary region of female nude mice with a 12-gauge trocar needle. When the tumors were 100–200 mm^3, the mice were pair-matched into treatment or control groups. Therapy was initiated on the day of pair-matching (day 1). NM-3 or vehicle (PBS) was administered to mice once daily by oral gavage (p.o.). Docetaxel or vehicle (2.5% polysorbate 80 and 1% ethanol in PBS) was injected into the tail vein on days 1, 5 and 9. NM-3 was administered 1 h before intravenous injection of docetaxel.

Statistical analyses

Statistical analyses were performed using GraphPad InStat software (GraphPad Software Inc.). Differences between groups were analyzed for statistical significance using one-way ANOVA followed by Dunnett's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

NM-3 decreases survival of NSCLC cells

To determine if NM-3 affects viability of human NSCLC cells, we first treated A549 cells with NM-3 at concentrations of 50–200 μg/ml for 72 h. Analysis of cell viability by the MTT assay demonstrated little if any effect (Fig. 1a). Similar results were obtained with Calu-6 cells (Fig. 1a). In addition, when assaying NCI-H23 cells, the effects of NM-3 on viability were significantly different from control, but modest in extent (Fig. 1a). To determine if longer exposures to NM-3 affect survival, the NSCLC cells were seeded and cultured in the presence of drug for 11–15 days. Colonies of 50 cells or greater were then stained and counted as a measure of clonogenic survival. Exposure of A549 cells to 10 μg/ml

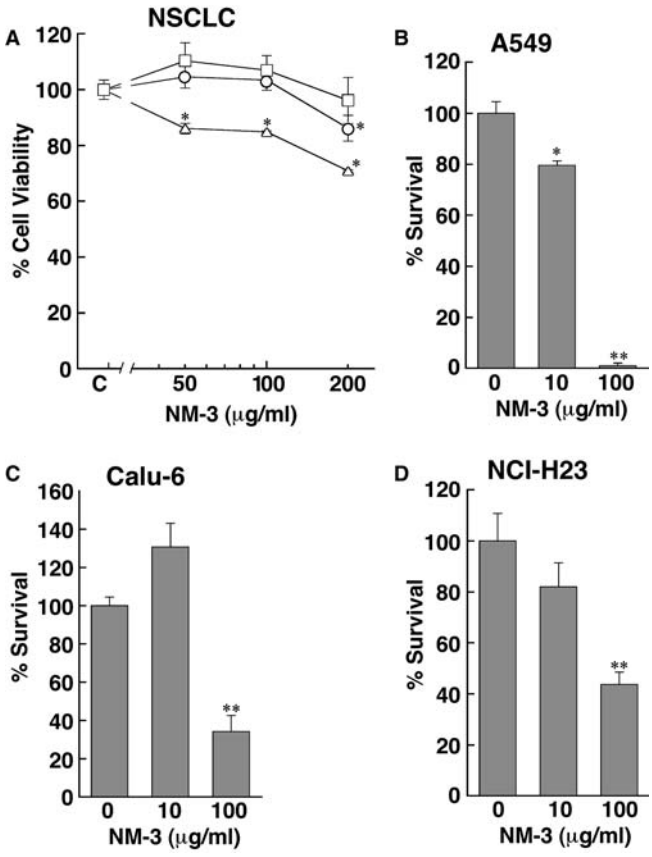

Fig. 1 NM-3 decreases survival of NSCLC cells in vitro. **a** A549 (○), Calu-6 (□) and NCI-H23 (△) cells were treated with the indicated concentrations of NM-3 for 72 h. cell viability was assessed by MTT assays. The results are expressed as the percentage of viable cells (mean ± SE of two independent experiments each performed in triplicate) compared to untreated cells. **b–d** A549 (**b**), Calu-6 (**c**) and NCI-H23 (**d**) cells were treated with the indicated concentrations of NM-3 for 11–15 days. The results are expressed as the percentage of colonies (mean ± SE of two independent experiments each performed in triplicate) compared to untreated cells. $^*P < 0.05$, $^{**}P < 0.01$ compared to control

NM-3 was associated with a limited decrease in survival (Fig. 1b). By contrast, treatment with 100 μg/ml NM-3 resulted in complete loss of A549 cell survival (Fig. 1b). The Calu-6 (Fig. 1c) and NCI-H23 (Fig. 1d) cells also responded to 100 μg/ml NM-3 with decreases in colony formation. These findings indicate that NM-3 decreases the survival of NSCLC cells after prolonged exposures to concentrations of 100 μg/ml.

NM-3 has no apparent effect on docetaxel activity against NSCLC cells in vitro

Docetaxel is an effective agent in the treatment of NSCLC [7]. To determine if NM-3 affects docetaxel-induced lethality of NSCLC cells in vitro, we first exposed A549 cells to different concentrations of NM-3 and docetaxel for 72 h. Assessment of viability demonstrated that docetaxel induces killing of A549 cells in

a concentration-dependent manner (Fig. 2a). Moreover, NM-3 at concentrations of 50–200 μg/ml had no apparent effect on docetaxel activity (Fig. 2a). Similar results were obtained when Calu-6 cells were exposed to NM-3 and docetaxel (Fig. 2b). As noted above, survival of NCI-H23 cells was modestly decreased by exposure to NM-3 (Fig. 2c). However, NM-3 had no additive effects on docetaxel-induced killing of NCI–H23 cells (Fig. 2c). These findings indicate that docetaxel activity against NSCLC cells in vitro is not potentiated by NM-3.

NM-3 is active against A549 cell xenografts

To determine if NM-3 has activity against NSCLC cells in vivo, we first treated mice bearing A549 tumor xenografts with NM-3 at an oral dose of 20 mg/kg/day for 25 days. Assessment of tumor volume demonstrated that NM-3 is effective in slowing growth of A549 tumors compared to the control (Fig. 3a). The effects of NM-3 were comparable to intravenous treatment of the A549 tumors with 1.25 mg/kg docetaxel on days 1, 5 and 9 (Fig. 3a). Moreover, the combination of NM-3 and docetaxel was more effective than either agent alone in inhibiting growth of A549 tumors (Fig. 3a). Similar results were obtained when treatment with NM-3 at 20 mg/kg/day for 25 days was combined with docetaxel at 5 mg/kg on days 1, 5 and 9 (Fig. 3b). To determine if NM-3 is more effective against A549 tumors at higher concentrations, we treated the tumor bearing mice with NM-3 at 100 mg/kg/day. The results show that the effects of 100 mg/kg/day NM-3 alone and in combination with docetaxel are comparable to those found with NM-3 dosing at 20 mg/kg/day (Fig. 3c). In concert with previous animal studies [3–5], the assessment of body weight as a surrogate marker of toxicity further showed that NM-3 is well-tolerated in mice bearing the A549 tumors (Fig. 3d). These findings indicate that NM-3 can potentiate the effects of docetaxel in the treatment of A549 tumor xenografts.

NM-3 also suppresses growth of NCI-H460 tumors

Other studies were performed on mice bearing NCI-H460 NSCLC tumor xenografts. NM-3 at a dose of 100 mg/kg/day failed to show activity alone or in combination with docetaxel (data not shown). Treatment with NM-3 at 500 mg/kg/day for 17 days significantly slowed growth of the NCI-H460 tumors (Fig. 4a). The antitumor effects of NM-3 as a single agent were similar to those found with docetaxel alone (Fig. 4a). In addition, the effects of NM-3 in combination with docetaxel were greater than either agent alone (Fig. 4a). Notably, 500 mg/kg/day NM-3 had no detectable effect on body weight compared to untreated tumor bearing mice (Fig. 4b). Moreover, NM-3 had no apparent effects on weight loss associated with docetaxel treatment (Fig. 4b). These findings indicate that NM-3 is effective alone and in combination with docetaxel in the treatment of NCI-H460 tumors.

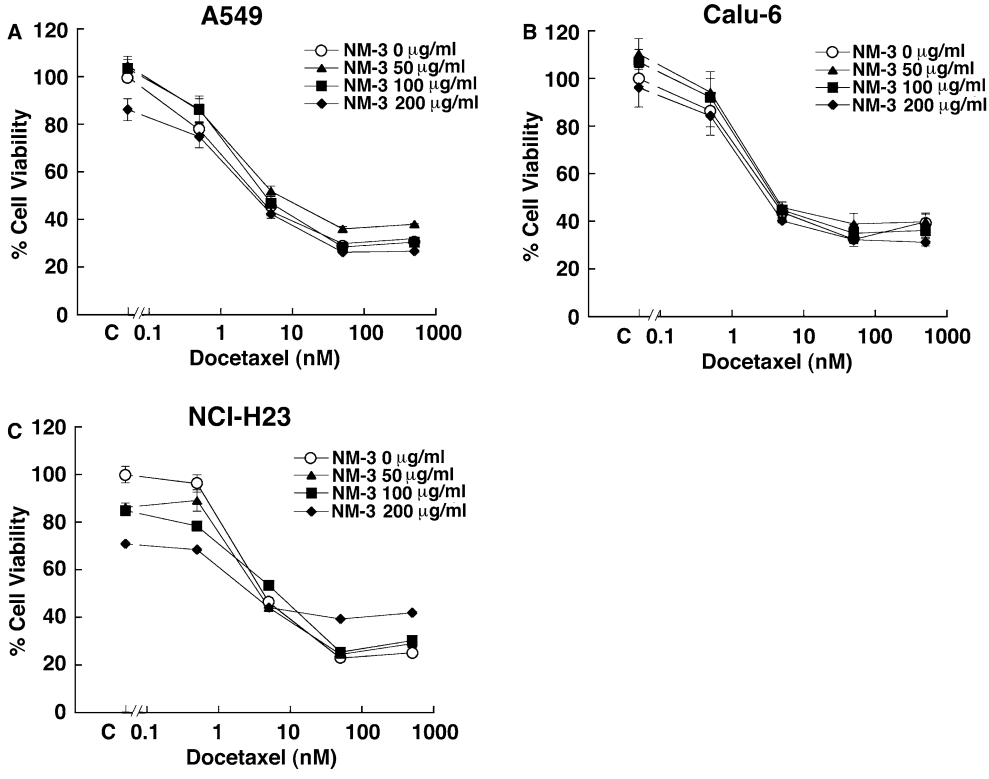

Fig. 2 Effects of NM-3 alone and in combination with docetaxel on viability of NSCLC cells in vitro. **a–c** A549 (**a**), Calu-6 (**b**) and NCI-H23 (**c**) cells were treated with the indicated concentrations of NM-3 and/or docetaxel for 72 h. Viability was determined by MTT assays. The results are expressed as the percentage of viable cells (mean ± SE of three determination) compared to control cells

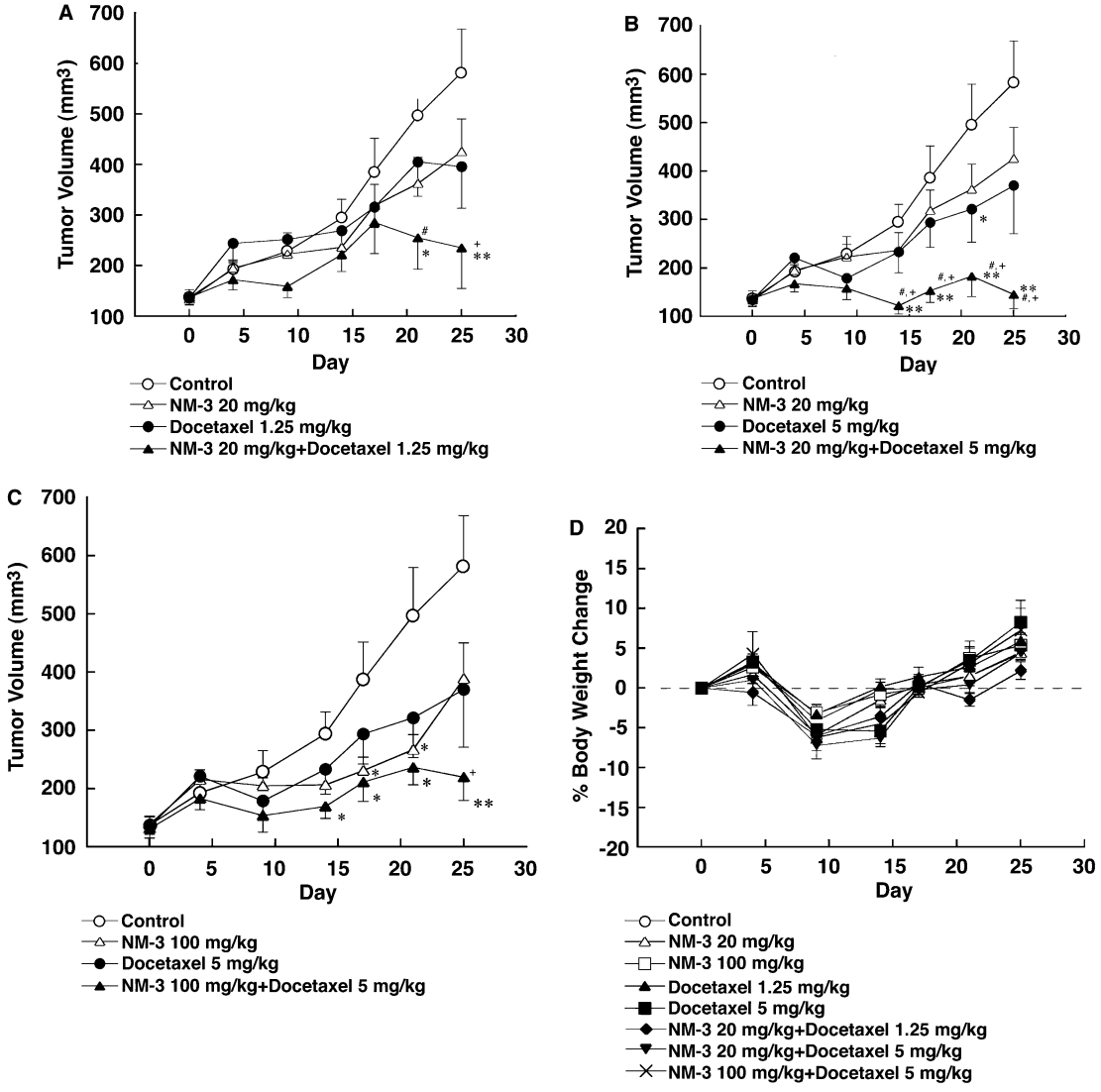

Fig. 3 Effects of NM-3 alone and in combination with docetaxel on growth of A549 tumor xenografts. **a–c** A549 tumor xenografts were treated with the indicated doses of NM-3 orally once-daily on days 1–25. Docetaxel was administered intravenously on days 1, 5 and 9. Tumor volumes are expressed as the mean ± SE for seven to eight mice in each group. $*P<0.05$, $**P<0.01$ compared to control.# and + represent statistical significance ($P<0.05$) compared to docetaxel alone and NM-3 alone, respectively. D. Body weight was determined for the treated A549 tumor bearing mice. The percentage body weight change is expressed as the mean ± SE for seven to eight mice in each group

NM-3 may be useful in the treatment of patients with NSCLC

NM-3 has recently completed Phase I evaluation as a single agent with oral dosing up to 1,500 mg/m^2 twice a day [1, 2]. Plasma levels that exceeded 100 mg/ml of NM-3 were achieved. Moreover, there was no dose-limiting toxicity, supporting the well-tolerated oral administration of this agent in man. The present studies demonstrate that NSCLC cell lines are sensitive to 100 mg/ml NM-3 in clonogenic survival assays. The results also show that NM-3 has single agent activity against A549 and NCI-H460 tumor xenografts. In anticipation of combining NM-3 clinically with cytotoxic anticancer agents, we asked if NM-3 exhibits activity with docetaxel, an agent used for the treatment of NSCLC [7]. In this regard, the activity of NM-3 in vivo may be attributable to anti-angiogenic effects on tumor endothelial cells, as well as antiproliferative and proapoptotic effects on tumor cells. NM-3 had no apparent effect on docetaxel-induced killing of NSCLC cells in vitro. However, NM-3 was effective in potentiating the activity of docetaxel in the treatment of A549 and NCI-H460 tumor xenografts. These findings could relate to effects of NM-3 on the tumor vasculature or to direct effects on tumor cells in vivo following prolonged (17–25 days) exposures. NM-3 may also have indirect effects on the tumor vasculature by suppressing VEGF levels as observed in diverse human cells [8] and in certain patients treated with NM-3 in the Phase I trials

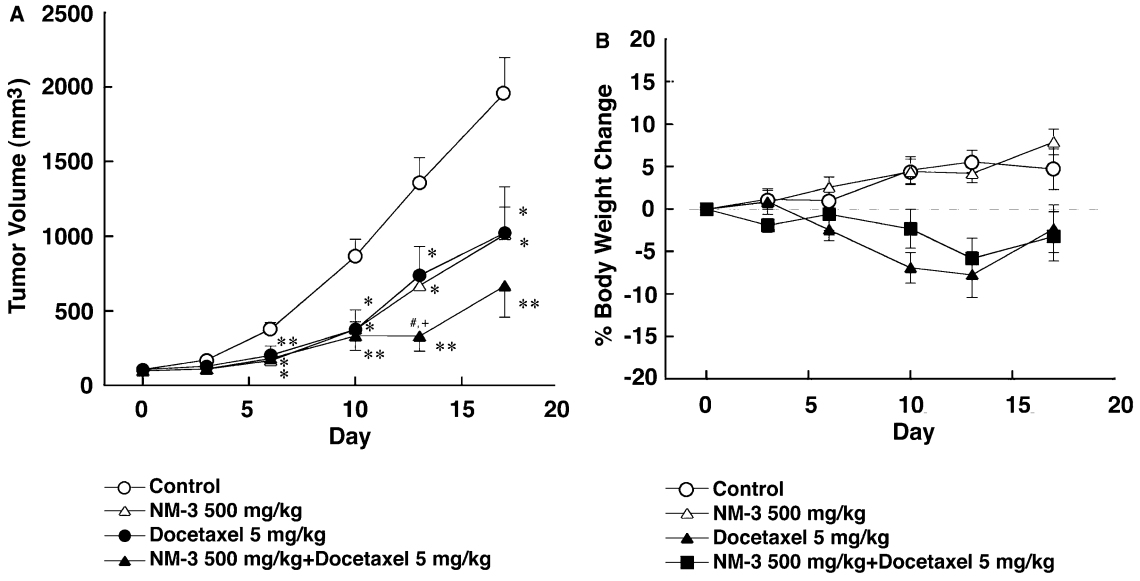

Fig. 4 NM-3 alone and in combination with docetaxel in the treatment of NCI-H460 tumor xenografts. **a** NCI-H460 tumor xenografts were treated with the indicated doses of NM-3 orally once-daily on days 1–17. Docetaxel was administered intravenously on days 1, 5 and 9. Tumor volumes are expressed as the mean ± SE for 10–19 mice in each group. $*P < 0.05$, $**P < 0.01$ compared to control.# and + represent statistical significance ($P < 0.05$) compared to docetaxel alone and NM-3 alone, respectively. **b** Body weight was determined for the treated NCI-H460 tumor bearing mice. The percentage body weight change is expressed as the mean ± SE for 10–19 mice in each group

[1, 2]. Thus, in certain patients, plasma VEGF levels may represent a surrogate marker of NM-3 activity. In summary, the oral bioavailability of NM-3, lack of significant toxicity and potential for enhancing docetaxel activity support the evaluation of NM-3 alone and in combination with docetaxel for the treatment of patients with NSCLC.

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